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Allozyme Types of Water Fern *Azolla japonica* and its Relatives (Azollaceae) Growing in Japan

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Samples of the aquatic floating fern *Azolla japonica* and its relatives (sect. *Azolla*) growing in 73 localities of Japan were analyzed by allozyme electrophoresis. Six allozyme types were recognized: AW, BW, BZ, TA, TO and YA types. Compared to the International Rice Research Institute (IRRI) accessions of *Azolla* by allozymes, the BZ and BW types had the same allozyme patterns as those of *A. caroliniana* and an artificial hybrid *Azolla*, respectively; It was supposed that both types had escaped from *Azolla* introduced to Japan for agricultural use on rice fields. The YA and AW types had very similar allozyme patterns to those of *A. filiculoides* and *A. microphylla*, respectively. The TA and TO types shared all allozyme bands except one. The TA and TO types were suggested to be closely related to *A. rubra* by allozyme comparison. Morphologically, it was suggested that numbers of septa in glochidia (spikelets on male massulae) differentiates the TA type from *A. rubra*, although only one sample of the TA type was observed. The TA (and possibly TO) type might be an independent species of genus *Azolla*, or true *A. japonica*.

Key words: allozyme, *Azolla japonica*, glochidium, introduced species

Azolla is an aquatic fern (Azollaceae; Pteridophyta) that is found floating on the water surface of small ponds and flooded rice fields, or growing on wet soil. According to Saunders & Fowler (1993), the genus *Azolla* is divided into two subgenera, subgen. *Azolla* and subgen. *Tetrasporocarpia*. The latter contains only one species, *A. nilotica* Decne. with a chromosome number of 2n=52 and clusters of four sporocarps. All species belonging to the former subgenus have choromosome number of 2n=44 and clusters of two sporocarps. Moreover, subgenus *Azolla* is separated into two sections, sect. *Azolla* and sect. *Rezosperma*, based on the morphology of megaspores and glochidia of male massulae, which are assemblages of microspores (Lumpkin & Plucknett 1980). Whole plant shapes and trichomes on stems are useful vegetative characteristics for distinguishing the two sections (Watanebe *et al.* 1992). Plant shape is ovate to fan-shaped and trichomes are only on leaves in sect. *Azolla*; the shape is deltoid to triangular and trichomes are on both stems and leaves in sect. *Rhizosperma*.

In Japan, two species, *Azolla japonica* Franch. et Sav. and *A. imbricata* (Roxb. ex Griff.) Nakai, have been recorded (Iwatsuki 1995). *Azolla japonica* belongs to sect. *Azolla*, and *A. imbricata* belongs to sect. *Rhizosperma*.

Currently, *Azolla japonica* has become rare,

perhaps due to the loss of its habitats and increased use of agricultural herbicides; it is listed as an endangered species in Japan (Environmental Agency of Japan 2000). On the other hand, *Azolla* is useful for agriculture, as its symbiotic cyanobacterium *Anabaena azollae* Strasb. can fix air nitrogen. Traditionally, *Azolla* has been used as a green manure for rice paddy fields in southern China and Vietnam (Moore 1969). The International Rice Research Institute (IRRI) in the Philippines has collected many accessions of *Azolla* from various parts of world and promoted utilization of selected *Azolla* strains as biofertilizer in rice paddy fields (Watanabe *et al.* 1987). In Japan, a rice-duck-*Azolla* system (combined use of ducks for weeding and *Azolla* as nitrogen nutrient for rice as well as food for ducks) on rice fields has been developed since 1993 and is now being rapidly adopted by organic farmers (Furuno 1997, 2001, Kishida & Utsunomiya 1998). Many stocks of exotic and artificial hybrid *Azolla* accessions from IRRI in the Philippines have been introduced for such use. It is possible that some have escaped or will escape and propagate vegetatively in nature. It is also necessary to check the effects in terms of behavior and genetic contamination of introduced *Azolla* upon native plants in Japan.

The taxonomical status of *Azolla japonica* is also controversial. Japanese botanists traditionally considered *A. japonica* endemic to Japan (Kurata & Nakaike 1987, Iwatsuki 1995). Some taxonomists have treated *A. japonica* as synonymous with *A. filiculoides* Lam. (Moore 1969, Lumpkin & Plucknett 1980). In an allozyme study for accessions of sect. *Azolla* cultivated at IRRI, Zimmerman *et al.* (1989) reported that *A. filiculoides*, *A. rubra* R. Br. and *A. microphylla* Kaulf. can be distinguished by their allozyme patterns. In their study, four Japanese accessions of *Azolla*, which had been treated as *A. rubra* at IRRI, were identified as *A. filiculoides*. However, they used only four accessions from Japan; other *Azolla* species might still exist in Japan, as Zimmerman *et al.* (1989) suggested.

For Japanese *Azolla*, Tanaka (1995) reported two distinct types of glochidia from *A. japonica*. Shiraiwa (2004) compared plants of *A. japonica* from Yamato-Koriyama City (Nara Pref.) with those from Toyo-oka City (Hyogo Pref.), and pointed out several morphological and ecological differences between them. These studies suggested the presence of several cryptic species among *A. japonica*. Characters of reproductive feature (micro-morphology of male massulae and macrospores) are very informative for species recognition of *Azolla*, but sporulation is so rare that it is difficult to observe these characters. Species identification by vegetative features is also difficult due to the plasticity and simplicity of *Azolla* morphology. For recognition and identification of cryptic species in ferns, molecular tools have been shown to be very helpful (Paris *et al.* 1989, Haufler *et al.* 1995, Yatabe *et al.* 2001). Our previous paper (Watanabe *et al.* 1999) suggested the presence of two cryptic species in *A. japonica* using random amplified polymorphic DNAs (RAPD) as markers.

In this study, allozyme markers are used to distinguish genetic types among *Azolla japonica* and its relatives naturally growing in Japan. The purpose of this paper was to reveal cryptic species in *A. japonica* and its relatives naturally growing in Japan and to clarify their taxonomical status using allozymes as genetic markers. We also discuss the situation arising from the induction of exotic *Azolla* for agriculture use.

Materials and Methods

Samples

Living plant materials of *Azolla japonica* and its relatives were collected from 73 localities in Japan shown as Fig.1 and Appendix 1. The fan shape of the plants was used for preliminary taxonomic identification of samples belonging to sect. *Azolla*. Some IRRI collections of *Azolla* were obtained from

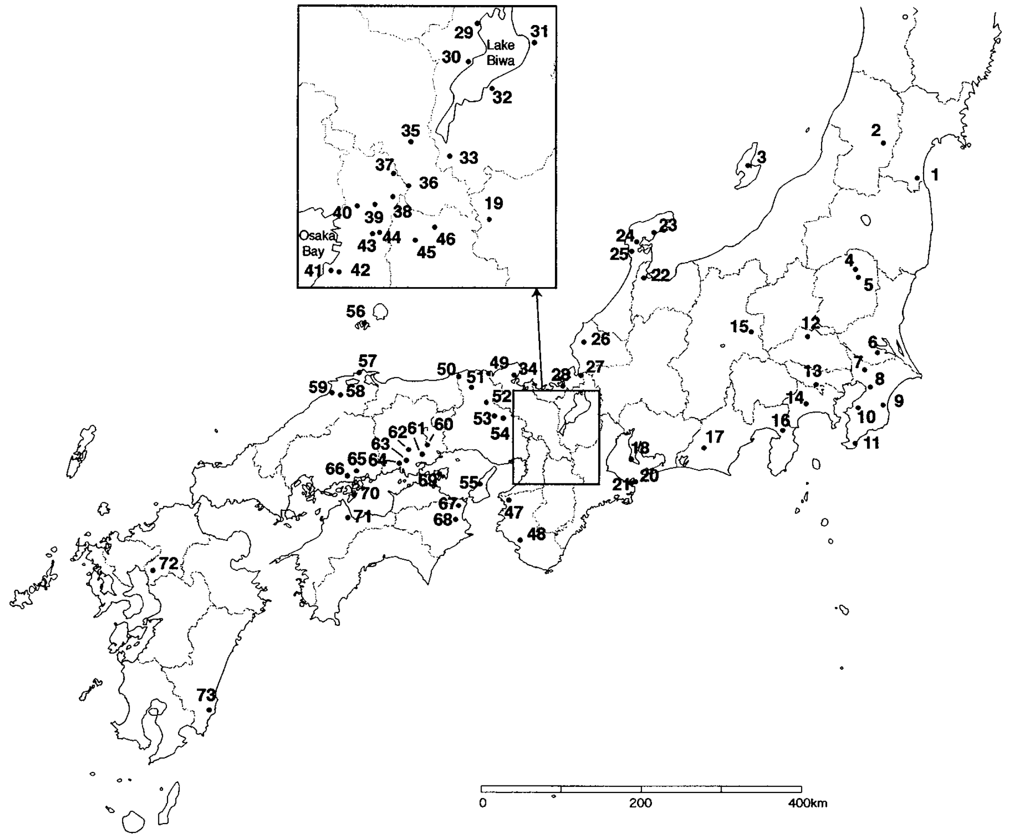

FIG. 1. Localities of plant materials of *Azolla japonica* and its relatives growing in Japan. Locality numbers correspond to Appendix 1.

Osaka Prefecture Univ. and/or Okayama Univ. as listed in Appendix 2: namely, FI1001 (*A. filiculoides* in IRRI list) and RU6502 (*A. rubra*), CA3004 (*A. caroliniana* Willd.), MI4138 (*A. microphylla*) and MI4087 (artificial hybrid between*A. microphylla* and *A. filiculoides*). Samples were cultivated at the greenhouse of the Museum of Nature and Human Activities, Hyogo by IRRI *Azolla* medium (Watanabe *et al.* 1992) and/or kept cool in plastic bags until use for experiments.

All vouchers were deposited in the herbarium of Nature and Human Activities, Hyogo (HYO).

Allozyme analysis

About 0.3g of total plants of *Azolla* were ground in 0.9ml of cold extraction buffer (Soltis *et al.* 1983); 0.1M Tris-HCl (pH.7.5), 1mM EDTA(4Na), 10mM KCl, 10mM MgCl2, 0.2%(v/v) 2-mercaptoethanol, and 5%(w/v) polyvinylpyrrolidone (Sigma 40T).

A total of eight enzymes were used: aspartate aminotransferase (AAT, E.C.2.6.1.1), aconitase (ACO, E.C.4.2.1.3), hexokinase (HK, E.C.2.7.1.1), isocitrate dehydrogenase (IDH, E.(C1.1.1.42), leucine aminopeptidase (LAP, E.C.3.4.11.1), phosphoglucoisomerase (PGI, E.C.5.3.1.9), phosphoglucomutase (PGM, E.C.2.7.5.1) and shikimate dehydrogenase (SKD, E.(C1.1.1.25). Electrophoresis was run on a horizontal starch gel system (Soltis *et al.* 1983) or vertical acrylamide slab gel system (Shiraishi 1988). The starch gel #8 buffer system of Soltis *et al.* (1983) was employed for HK, LAP and PGI; the #10 system of Soltis *et al.* (1983) was used for ACO and SKD; Histidine buffer system for starch gel (Cardy *et al.* 1981) was used for IDH and PGM. AAT was resolved in an acrylamide system (Shiraishi 1988).

Staining procedures for these enzymes followed Wendel & Weeden (1989). Loci were num-

bered with the most anodal form as "*1*" and so on when more than one present for an enzyme. Alleles were labeled at each loci, with the most anodal form designated "*a*" and progressively slower forms as "*b*","*c*" and so on. In cases where it was difficult to distinguish loci, the most anodal band was designated "A" and progressively slower bands as "B","C" and so on.

Preliminary observation of glochidia of male massulae

Samples of male massulae for glochidium observation were mainly obtained from cultivated plants at the greenhouse. Samples of locality No.43, 62 and 63 were obtained from natural populations. Glochidia of male massulae of *Azolla* were also examined using herbarium specimens kept in the herbaria of Kobe Gakuin Univ., Kyoto Univ. (KYO), Osaka Museum of Natural History (OSA) and Univ. of Tokyo (TI). Glochidia were observed

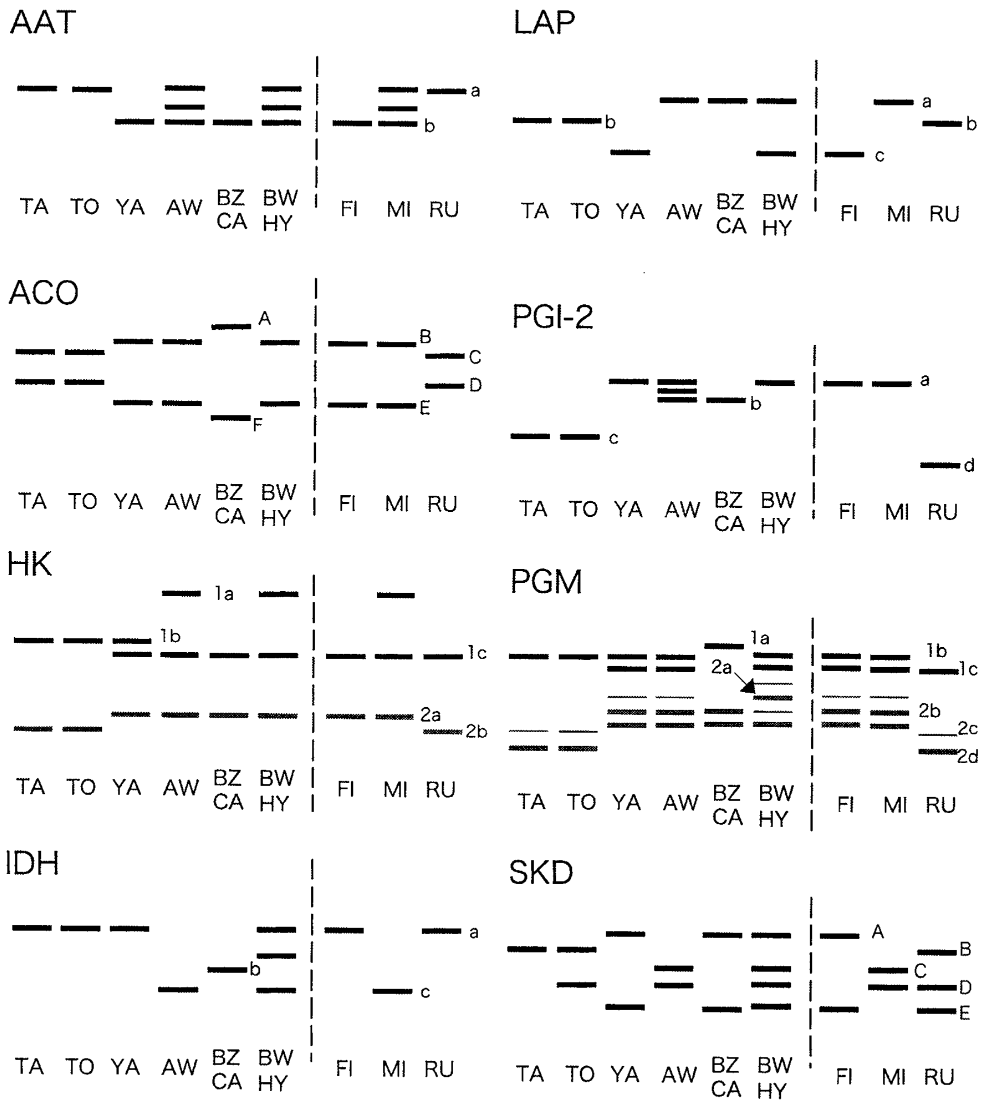

FIG. 2. Schematic zymograms of six allozyme types of *Azolla japonica* and its relatives and IRRI Azolla accessions examined in this study. The anodal side is the upper side of this figure. At HK and PGM, black bands are interpreted as HK-1 and PGM-1; gray bands are interpreted as HK-2 and PGM-2. In PGM-2, fainter bands (thin line) are treated as ghost bands. CA, FI, HY, MI and RU are abbreviations for IRRI CA 3004, FI1001, MI4087 (artificial hybrid), MI4138 and RU6502, respectively. See text for other six abbreviations to allozyme types.

TABLE 1. Alleles or bands of six allozyme types of *Azolla japonica* and its relatives in Japan together with IRRI *Azolla* accessions examined in this study. Alleles are listed in *Italic*, bands in Roman. Abbreviations for allozyme types and IRRI accessions correspond to those of Fig. 2.

	Aat	*Hk-1*	*Hk-2*	*Idh*	*Lap*	*Pgi-2*	*Pgm-1*	*Pgm-2*	ACO	SKD
TA	*a*	*b*	*b*	*a*	*b*	*c*	*b*	*d*	CD	BD
TO	*a*	*b*	*b*	*a*	*b*	*c*	*b*	*d*	CD	B
RU	*a*	*c*	*b*	*a*	*b*	*d*	*c*	*d*	CD	BCD
YA	*b*	*bc*	*a*	*a*	*c*	*a*	*bc*	*bc*	BE	AE
FI	*b*	*c*	*a*	*a*	*c*	*a*	*bc*	*bc*	BE	AE
AW	*ab*	*ac*	*a*	*c*	*a*	*ab*	*bc*	*bc*	BE	CD
MI	*ab*	*ac*	*a*	*c*	*a*	*a*	*bc*	*bc*	BE	CD
BZ	*b*	*c*	*a*	*b*	*a*	*b*	*a*	*bc*	AF	AE
CA	*b*	*c*	*a*	*b*	*a*	*b*	*a*	*bc*	AF	AE
BW	*ab*	*ab*	*a*	*ac*	*ac*	*a*	*bc*	*ac*	BE	ACDE
HY	*ab*	*ab*	*a*	*ac*	*ac*	*a*	*bc*	*ac*	BE	ACDE

by light microscope (OLYMPUS, HB-2 model). The number of septa in each glochidium was counted in more than five glochidia each of more than seven male massulae.

Results

Allozyme analysis

Zymograms and interpreted alleles are shown in Fig. 2 and Table1. Stergianou & Fowler (1990) reported that most accessions of *Azolla japonica* and its relatives (species of sect. *Azolla*) were cytologically diploid and that the three accessions from Japan as well as IRRI FI1001, CA3004 and RU6502 used in this study were also diploid. Therefore, sample plants were assumed to be diploid. Isozyme numbers or band numbers were consistent with those of a typical diploid plant. Eight putative loci of six enzymes were scorable and polymorphic: *Aat*, *Hk-1*, *Hk-2*, *Idh*, *Lap*, *Pgi-2*, *Pgm-1* and *Pgm-2*. For ACO and SKD, only band patterns were shown in Fig. 2 and Table1 because zymograms could not be interpreted to loci and genotypes. For PGM-2, fainter bands were detected and treated as ghost bands (thin bands at PGM-2 in Fig. 2).

Six allozyme types (TA, TO, YA, AW, BZ and BW) were recognized in Japanese *Azolla*. These types were first found at localities No. 49 (Tajima Prov.), No. 13 (Tokyo Pref.) No. 45 (Yamato Prov.), No. 67 (Awa Prov.), No. 60 (Bizen Prov.), No. 32 (Lake Biwa shore), and were named after these locality names respectively. The geological distribution of the six allozyme types is shown in Fig. 3.

The TA and TO types shared all allozyme alleles or bands except for a D band at SKD. Included in these were many unique alleles or bands: $Hk\text{-}2^b$, Lap^b, $Pgi\text{-}2^c$, $Pgm\text{-}2^d$, CD bands at ACO, and B or BD bands at SKD. Among the compared IRRI accessions, RU6502 shared the largest number of alleles (Aat^a, $Hk\text{-}2^b$, Lap^b, $Pgm\text{-}2^d$) and bands (CD at ACO) with them. The TA type was found at 36 localities in Honshu and Shikoku; the TO type was found at 7 localities around Kanto District in Honshu.

The YA type showed the same zymogram patterns as those of IRRI FI1001 at all loci except *Hk-1*. This type was distributed through 19 localities of Honshu (mainly the western part) and Kyushu.

The BW and BZ types shared all alleles or bands with the IRRI MI 4087 and CA2038, respec-

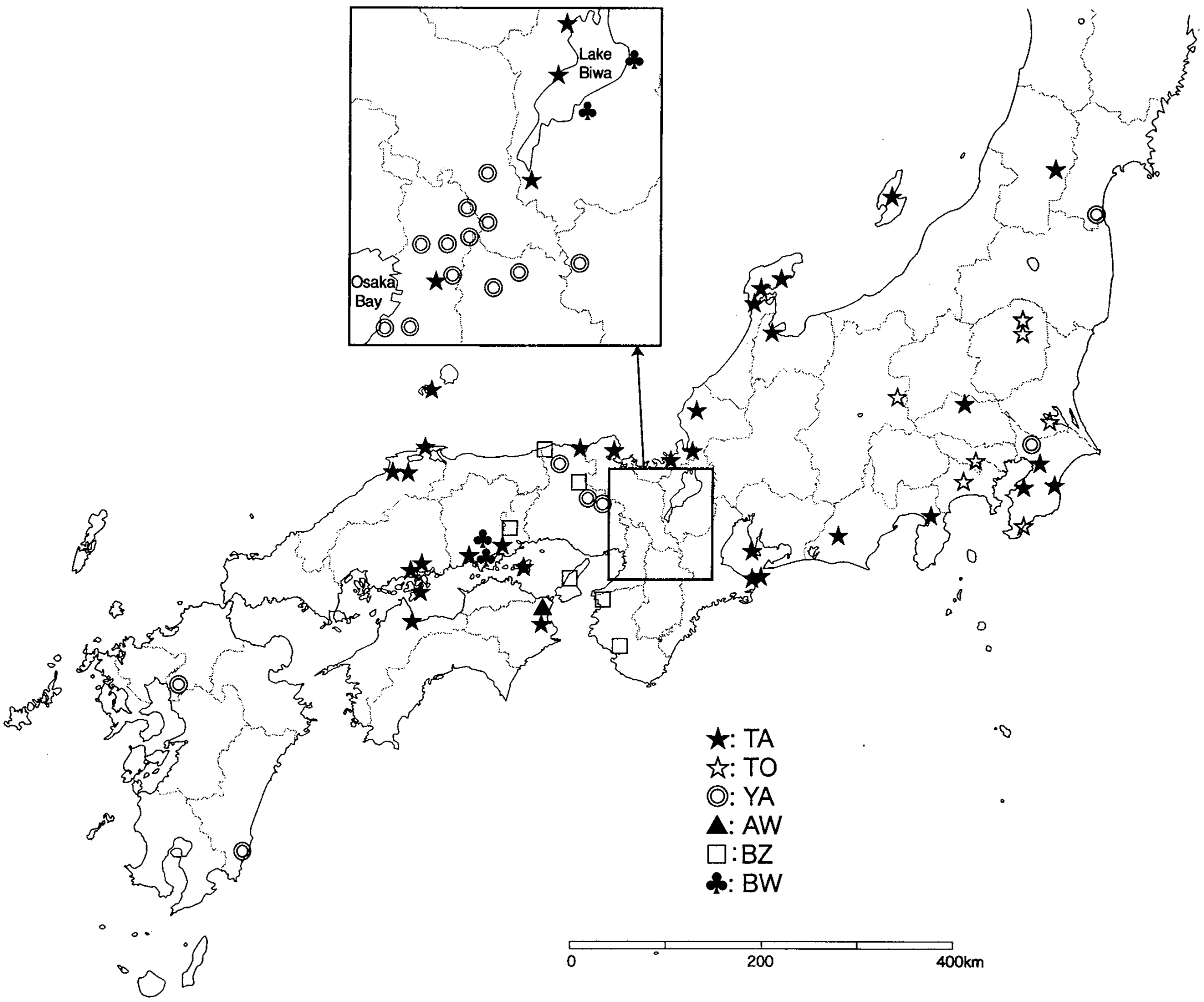

FIG. 3. Geographical distribution of six allozyme types of *Azolla japonica* and its relatives in Japan.

tively. The AW type had the same alleles or bands as those of IRRI MI4138 except at the *Pgi-2* loci. The BW and BZ types were found in the western part of Honshu. The AW type was found in only one locality of Shikoku (locality No. 58).

Preliminary Observation of glochidia of male massulae

Photographs of glochidium (spikelet structures on surface of male massulae) are shown in Fig. 4. Mean numbers of septa for each glochidium of allozyme samples of *Azolla* are shown in Table 2, those of herbarium specimens in Table 3.

Some plants of the YA type from 10 localities produced sporangia in early summer of 1995-2001 in the greenhouse. Their glochidia had mainly 0-1 septa (Fig. 4A, Table 2). Male massulae of the BW type were obtained in late autumn of 2002 in the field at two localities, and their glochidia had 0-1, but rarely 2 septa like those of the YA type (Fig. 4B & Table 2). Glochidia of male massulae of IRRI FI1001 (*Azolla filiculoides*) were equipped with mainly one, sometimes no or two septa (Fig. 4C & Table 2).

Microsporocarps of the TA type were obtained only from Higashi-Osaka, Osaka Pref. (locality No.43) on June of 1995. More than three septa were present at the upper part of glochidia (Fig.

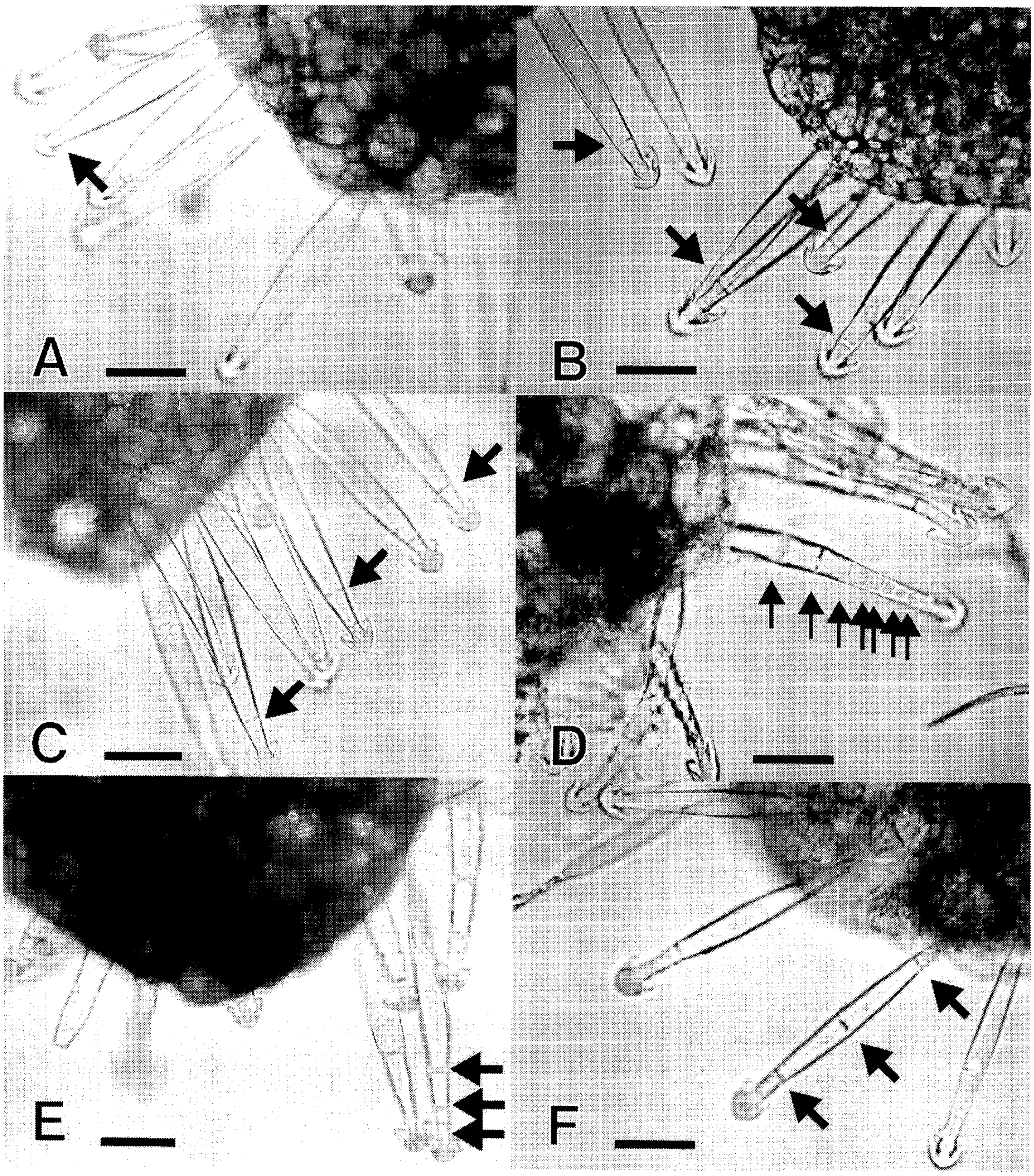

FIG. 4. Glochidia of *Azolla*. An arrow indicates each septum in glochidium. A, YA type, Kyoto Pref., Kyo-tanabe City, Takigi (locality No. 36); B, IRRI FI1001 (*A. filiculoides*); C, TA type, Osaka Pref., Higashi-osaka City, Shukubo-cho,No.2 (locality No. 43); D, Herbarium specimen of *A. rubra*, New Zealand (see Table 3); E, Herbarium specimen of *A. japonica*, Tokyo Pref., Shimura, (see Table 3); F, BW type, Okayama Pref., Kurashiki City, Futako (locality No.63). Scale bars = 20μm.

4D & Table 2). Eight herbarium specimens of *Azolla japonica* kept at the herbaria of Kyoto Univ. (KYO) and Osaka Museum of Natural History (OSA) had glochidia with several septa in the upper parts, like those of the TA type (Fig. 4E & Table 3). A specimen of *A. rubra* at the herbarium of Kobe Gakuin University showed glochidia with several septa at constant intervals (Fig. 4F & Table 3).

Discussion

This study revealed the presence of six allozyme types in *Azolla japonica* and its relatives (sect. *Azolla*) naturally growing in Japan. Our previous

TABLE 2. Numbers of septa for each glochidium on *Azolla* examined in this study. Allozyme types correspond to those of Fig. 2 and Appendix 1.

Allozyme type	No.	Locality	No. of septa mean±SD	No. of examined glochidia (massulae)
YA	1	MIYAGI, Yamamoto	0.08±0.22	84 (7)
YA	7	CHIBA, Shonan	0.18±0.28	77 (7)
YA	19	MIE , Ueno	0.16±0.32	103 (7)
YA	36	KYOTO, Kyo-Tanabe	0.13±0.36	158 (10)
YA	38	OSAKA, Katano	0.01±0.30	132 (10)
YA	39	OSAKA, Neyagawa	0.17±0.33	100 (7)
YA	40	OSAKA, Settsu	0.14±0.37	128 (10)
YA	44	OSAKA, Higashi-osaka	0.05±0.25	166 (10)
YA	45	NARA, Yamato-koriyama	0.03±0.15	104 (7)
YA	46	NARA, Nara City	0.23±0.43	137 (7)
BW	62	OKAYAMA, Okayama City	0.35±0.42	103 (7)
BW	63	OKAYAMA, Kurashiki	0.42±0.52	123 (7)
TA	43	OSAKA, Higashi-osaka	4.14±1.30	43 (8)
IRRI accession				
FI1001		East Germany	0.96±0.47	131 (10)

TABLE 3. Numbers of septa for each glochidium observed in herbarium specimens of *Azolla*.

Locality	Collector	Date	No. of septa mean±SD	No. of examined glochidia (massulae)
A. japonica				
IBARAGI Pref.				
Ibaragi Town, Naka-ishizaki	Noguchi s.n.(OSA)	Jun. 25,1979	6.00±1.52	53 (7)
Kita-ibaragi City, Minami-nakago	Yasu 454390.(OSA)	Sep.12,1979	3.47±1.37	49 (8)
CHIBA Pref.				
Sakura City, Oo-sakura	Namegata 2364.(OSA)	Jul. 03,1952	3.95±0.98	58 (7)
Chosei Town	Tagawa 8683.(OSA)	May 29,1962	4.62±1.02	102 (9)
TOKYO Pref.				
Itabashi-ku, Shimura	Makino s.n. (OSA)	May 11,1919	3.37±1.16	83 (10)
Oota-ku, Ooimachi	Makino s.n. (KYO)	May 9,1919	3.55±1.20	42 (7)
KYOTO Pref.				
Kyoto City, Takaragaike	Tagawa 3844(KYO)	Jul. 19,1951	5.70±2.02	83 (10)
OKAYAMA Pref.				
Okayama City, Takamatsu	Tsuboi s.n. (KYO)	Jun.10,1937	3.95±0.98	58 (7)
A. rubra				
NEW ZEALAND				
Mario Channel near Blenheim	Mason 3109 (KGU[a])	Jan.21,1955	2.54±0.71	61 (7)

[a] herbarium of Kobe Gakuin University

paper using RAPD analysis showed two distinct groups in *A. japonica* (Watanabe *et al.* 1999). All samples designated as group A (including *A. filiculoides*) belonged to the YA type, and samples of group B were divided into TA and TO types (Appendix 1).

BZ type and BW type showed completely identical allozyme patterns to IRRI CA3004, which was identified as *Azolla caroliniana*, and IRRI MI4087, which was an artificial hybrid between *A. microphylla* and *A. filiculoides*, respectively. The rice-duck-*Azolla* system has been applied in Japan since 1993 (Furuno 1997). Some IRRI *Azolla* accessions, including both CA3004 and MI 4087, have been provided to Japanese farmers from Okayama Univ. (Kishida pers. com.). In the prefectures where BW and BZ types were found (Shiga, Wakayama, Hyogo, Okayama), many organic farmers have used the rice-duck-*Azolla* system since 1997 (Kishida pers. com.). It was recorded that an organic farmer of locality No.50 has been supplied IRRI *Azolla* accession CA3004 from Prof. Shiomi, Osaka Prefecture Univ. and has used it on his rice field since 1993 (Shiomi pers. com.). BZ type reported from locality No.50 in this study were collected in a rice paddy field 8km apart from the organic farmer's rice field in 2000 (Hamasaka construction office, Hyogo Pref. pers. com.). It is highly likely that at least some of the BZ and BW types found in this study were plants that had escaped from rice paddies and become naturalized in Japan.

The YA type showed nearly the same zymograms as those of IRRI FI1001, which was identified as *Azolla filiculoides*, and the AW type showed very similar zymograms to those of IRRI MI4138, which was labeled as *A. microphylla*. It would be reasonable for the YA and AW types to be treated as *A. filiculoides* and *A. microphylla*, respectively, based on the results of allozyme analysis. Zimmerman *et al.* (1989) identified four IRRI accessions originating from Japan as *A. filiculoides*. They might have detected only the YA type from Japan.

Glochidia with no or one septa were observed in ten samples among all nineteen samples of the YA type, including that from locality No. 40 (Osaka Pref., Settsu City), where Tanaka (1995) observed the same type of glochidia in 1994. As for the morphology of glochidia, that of IRRI FI1001 (*Azolla filiculoides*) was similar to that of the YA type. These finding were consistent with previous reports about *A. filiculoides* by Svenson (1944).

The TA and TO types shared almost the same alleles or bands、 which suggested that TA and TO types are very closely related. Plants of TO type were found only around Kanto District, and the difference at SKD might be due to geographical variation. YA type and IRRI FI1001 (*Azolla filiculoides*) showed no similar allozyme patterns to those of the TA and TO types. Among IRRI accessions examined in this study, IRRI RU6502 (identified as *A. rubra*) had the allozyme pattern most similar to those of TA and TO types. We considered that the TA and TO types are more closely related to *A. rubra* than to *A. filiculoides*. Whether or not the TA and TO types should be attributed to *A. rubra*, it would not be accurate to attribute all Japanese native *Azolla* to A. filiculoides.

Only one sample of male massulae of the TA type was obtained at the irrigation pond in Higashiosaka City, Osaka Pref. (locality No. 43). Several septa were present, mainly in the upper parts of its glochidia (Fig. 4C). Tanaka (1995) observed glochidia with several septa on samples from the same pond. Glochidia with several septa in upper part were also observed in some herbarium specimens collected between 1919 and 1979 (Fig. 4E, Table 3). Seto & Nasu (1975) reported the same type of glochidia observed in the specimen kept in OSA (Tagawa 8683), which was also examined in this study (see Table 3). The existence of herbarium specimens with this glochidium morphology suggest that the TA type (and possibly the TO type) has been present in Japan at least since 1919.

Unfortunately, we could not find glochidium

with no or one septa among herbarium specimens in this study. Hence, there is no information about YA type before 1984, when Lumpkin has obtained IRRI FI 1603 (identified as *Azolla filiculoides*) from Kyo-tanabe City, Kyoto Pref. (Watanabe *et al.* 1992).

Glochidia of *Azolla rubra* were observed to have several septa at constant intervals (Fig.4F), and they are clearly different from those of the TA type (Fig.4D). Chinnock (1998) and Large & Bragginns (1993) also has shown glochidia with several septa at constant intervals on male massulae of Australian and New Zealand *A. filiculoides*, which should be treated as *A. rubra* by the taxonomical treatment of Zimmerman *et al.* (1989). Allozyme data and glochidium morphology suggested that the TA (and possibly the TO) type is neither *A. filiculoides* nor *A. rubra* and should be treated as an independent species. A type specimen of *A. japonica* was collected in Yokosuka (Kanagawa Pref., Kanto Dist.) by Savatier in 1866-1876 when he stayed in Japan (Franchet & Savatier 1876). Herbarium specimens of *Azolla* suggest that *Azolla* with several septa had been distributed in Kanto District on the time of Savatier's stay in Japan. The TA (and possibly the TO) type might be true *A. japonica*. Additional research, including analysis of cpDNAs and other morphological characters, are necessary to make a conclusion on the taxonomical status of each allozyme type found in this study, especially for the TA and TO types.

The Japanese government and regional governments in Japan have listed *Azolla japonica* in their Red Data Books. However, "*A. japonica*" in their list might be a mixture of several allozyme types of *Azolla*. For the conservation of native Japanese *Azolla*, each allozyme type should be distinguished separately in the Red Data Books.

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APPENDIX 1. Localities, collectors, allozyme types, glochidium types, RAPD groups of samples of *Azolla japonica* and its relatives examined in this study.

No.	Locality	Collector (Voucher No. in HYO)	Allozyme type[a]	RAPD group[b]
	HONSHU			
1	Miyagi Pref., Yamamoto Town, Kodaira	T.Usuba (C1-168001)	YA	-
2	Yamagata Pref., Higashine City, Nikuchi	K.Sawa (C1-168002)	TA	-
3	Niigata Pref., Sado City	M.Sasagawa (C1-168003)	TA	-
4	Tochigi Pref., Ootawara City, Ichinozawa	S.Waku (C1-133188)	TO	-
5	Tochigi Pref., Kuroiso City, Nabekake	S.Waku (C1-133187)	TO	-
6	Ibaragi Pref., Tsuchiura City, Teno-cho, Ishida	S.Matsumoto (C1-131327)	TO	-
7	Chiba Pref., Shonan Town, Yanagito	M.Shimosegawa (C1-168004)	YA	-
8	Chiba Pref., Chiba City,Wakaba-ku, Ookusa-cho	T.Shimakawa (C1-116168)	TA	-
9	Chiba Pref., Ichinomiya Town, Higashi-namimi	K.Nakamura (C1-168005)	TA	B
10	Chiba Pref., Sodegaura City, Kami-izumi	O.Kawana (C1-168006)	TA	B
11	Chiba Pref., Chikura Town, Mt.Takatsuka	T.Yamada (C1-116185)	TO	B
12	Saitama Pref., Kumagaya City, Kuge	S.Takahashi (C1-131631)	TA	-
13	Tokyo Pref., Machida City, Zushi-cho, Gotanda	A.Yamamoto (C1-133190)	TO	-
14	Kanagawa Pref., Hadano City, Toge	J.Nagaoka (C1-133189)	TO	-
15	Nagano Pref., Usuda Town, Jizou-ike pond	H.Shimidu (C1-168007)	TO	-
16	Shizuoka Pref., Shimizu Town, Kakita-gawa	I.Sasamoto (C1-168008)	TA	-
17	Shizuoka Pref., Hamakita City, Nishinotani	K.Nakamura (C1-131333)	TA	-
18	Aichi Pref., Mihama Town, Okuda	I.Watanabe (C1-116177)	TA	B
19	Mie Pref., Ueno City, Kami-Tomoo	K.Yamakawa (C1-168009)	YA	-
20	Mie Pref., Toba City, Toshi Isl., Nasa	I.Watanabe (C1-116171)	TA	-
21	Mie Pref., Toba City, Toshi Isl., Momotori	I.Watanabe (C1-116173)	TA	B
22	Toyama Pref., Takaoka City, Zukawa	T.Suzuki (C1-133262)	TA	-
23	Ishikawa Pref., Noto Town, Hanami	T.Suzuki (C1-131328)	TA	B
24	Ishikawa Pref., Nakajima Town, Besho	T.Kawahara (C1-116181)	TA	-
25	Ishikawa Pref., Shiga Town, Tsubono,	T.Suzuki (C1-133268)	TA	-
26	Fukui Pref., Fukui City, Higashi-shinmachi	Y.Saito (C1-131548)	TA	-
27	Fukui Pref., Tsuruga City, Nakaikemi	M.Maeda (C1-116182)	TA	-
28	Fukui Pref., Mikata Town, Tai	A.Hirayama (C1-168044)	TA	-
29	Shiga Pref., Makino Town, Nishihama	T.Suzuki (C1-168010)	TA	-
30	Shiga Pref., Takashima Town, Otomegaike	A.Murata (C1-168011)	TA	-
31	Shiga Pref., Hikone City, Yasaka-cho, Inugami River	A.Murata (C1-168012)	BW	-
32	Shiga Pref., Nakasu Town, Yoshikawa	A.Murata (C1-168013)	BW	-
33	Shiga Pref., Otsu City, Ooishi-Sotsuka	M.Kanda (C1-116174)	TA	-
34	Kyoto Pref., Miyazu City, Komatsu	T.Suzuki (C1-116166)	TA	-
35	Kyoto Pref., Kyoto City, Fushimi-ku, Daigo-sanpo Temple	T.Suzuki (C1-168042)	YA	-
36	Kyoto Pref., Kyo-tanabe City, Takigi	T.Suzuki (C1-168043)	YA	-
37	Osaka Pref., Hirakata City, Son-enji	S.Fujii (C1-116186)	YA	-
38	Osaka Pref., Katano City, Morikita 2chome	T.Suzuki (C1-168014)	YA	-
39	Osaka Pref., Neyagawa City, Umegaoka 2 chome	T.Suzuki (C1-168015)	YA	-
40	Osaka Pref., Settsu City, Senrugaoka 2 chome	T.Suzuki (C1-116178)	YA	-
41	Osaka Pref., Sakai City, Daisen Park	T.Suzuki (C1-168016)	YA	-
42	Osaka Pref., Sakai City, Ooizumi Park	T.Suzuki (C1-168017)	YA	A
43	Osaka Pref., Higashi-osaka City, kyakubo-cho, #2	J.Takeuchi (C1-129553)	TA	-
44	Osaka Pref., Higashi-osaka City, kyakubo-cho, #3	J.Takeuchi (C1-129554)	YA	A
45	Nara Pref., Yamato-koriyama City, Tenjo-cho	T.Shiraiwa (C1-168018)	YA	-

APPENDIX 1. (continued)

No.	Locality	Collector (Voucher No. in HYO)	Allozyme type[a]	RAPD group[b]
46	Nara Pref., Nara City, Nara Park	T.Fujii (C1-116169)	YA	-
47	Wakayama Pref., Wakayama City, Uchihara	A.Naito (C1-168019)	BI	-
48	Wakayama Pref., Tanabe City,Kagihara	K.Nakano (C1-168020)	BI	-
49	Hyogo Pref., Toyo-oka City, Tai	T.Suzuki (C1-063295)	TA	B
50	Hyogo Pref., Hamasaka Town, Igumi	Y.Kadono (C1-168021)	BI	-
51	Hyogo Pref., Muraoka Town, Itashino	M.Sakoda (C1-168022)	YA	-
52	Hyogo Pref., Yabu City, Yoka-machi, Isa,	Y.Sawada (C1-168023)	BI	-
53	Hyogo Pref., Hikami Town, Kinuyama	T.Suzuki (C1-145941)	YA	-
54	Hyogo Pref., Kasuga Town, Furukawa	T.Suzuki (C1-168024)	YA	-
55	Hyogo Pref., Goshiki Town, Ayuhara	T.Suzuki (C1-168025)	BI	-
56	Shimane Pref., Oki, Nishinoshima, Mimiura	S.Kariyama (C1-168026)	TA	-
57	Shimane Pref., Shimane Town, Oashi	Y.Sugimura (C1-168027)	TA	B
58	Shimane Pref., Izumo City, Tokorohara-cho	Y.Tsujii (C1-168028)	TA	-
59	Shimane Pref., Koryo Town, Sashimi	Y.Sugimura (C1-131334)	TA	-
60	Okayama Pref., San-yo Town, Toyu	T.Suzuki (C1-168029)	BI	-
61	Okayama Pref., Okayama City, Omachi	H.Kobatake (C1-116179)	TA	B
62	Okayama Pref., Okayama City, Ashimori	T.Suzuki (C1-168030)	BW	-
63	Okayama Pref., Kurashiki City, Futako	S.Kariyama (C1-168031)	BW	-
64	Okayama Pref., Kurashiki City, Tsurashima-cho, Yagake	C.Kaibara (C1-168032)	TA	-
65	Hiroshima Pref., Mihara City, Koizumi-cho, Akishige	T. Sano (C1-168033)	TA	-
66	Hiroshima Pref., Takehara City, Fukuda-cho, Daijo	T. Sano (C1-168034)	TA	-
	SHIKOKU			
67	Tokushiam Pref., Naruto City, Ootsu-cho, Daiko	T.Tabuchi (C1-116176)	AW	-
68	Tokushima Pref., Tokuahima City, Iitani-cho, Nagatani	S.Kinoshita (C1-168035)	TA	-
69	Kagawa Pref., Tonosho Town, Uneki	O.Kume (C1-168036)	TA	-
70	Ehime Pref., Hakata Town, Ookoe	M.Fujita (C1-168037)	TA	-
71	Ehime Pref., Saijo City, Nishi-teizui	M.Fujita (C1-116183)	TA	B
	KYUSHU			
72	Fukuoka Pref., Ooki Town, Fukuma	T.Shogomori (C1-168038)	YA	A
73	Miyazaki Pref., Nichinan City	Y.Saiki (C1-168039)	YA	-

[a]Allozyme types correspond to those of Fig.2.

[b]A and B are referred to group A and B of Watanabe *et al.*(1999), respectively.

APPENDIX 2. IRRI *Azolla* accesions examined in this study

IRRI No.	Species	Locality or Origin	Voucher No. in HYO
FI1001	*A. filiculoides*	East Germany	C1-131412
CA3008	*A. caroliniana*	Urguay	C1-168040
MI4087	hybrid	artificial hybrid between *A. microphylla* and *A. filiculoides*	C1-168041
MI4138	*A. microphylla*	China	C1-168045
RU6502	A. rubra	Australia, Victoria	C1-116167